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Title:

MILK POWDERS AND SIMILAR PRODUCTS

DETERMINATION OF β-LACTOGLOBULIN BY ELISA

LI number
08.084-1

References:
NRC/QS/
MHc/HWt/cre

Quality criteria: β-lactoglobulin

1 SCOPE AND FIELD OF APPLICATION

Description of a method for the determination of β-lactoglobulin antigenicity in products containing milk, whey and casein hydrolysates, and for detection of milk and whey protein contaminations in infant cereals, soya based infant formulas and similar products.

2 DEFINITIONS AND ABBREVIATIONS

2.1 Definitions

β-lactoglobulin: protein of bovine milk, representing about 50% of total whey proteins (about 3 g/kg full cream milk). In milk, it is present as a dimer with a molecular mass per monomer of 18283 Da.

2.2 Abbreviations

BLG : β-lactoglobulin
ELISA : enzyme-linked immunosorbent assay
TMB : Tetramethylbenzidine

3 PRINCIPLE OF THE METHOD

The determination of β-lactoglobulin antigenicity is made using an indirect competitive ELISA.

Samples are dissolved in water or in diluent buffer and diluted with the same buffer. The immunoassay is performed in plastic microwells, which have been precoated with the antigen BLG.

In the initial competition reaction, the diluted sample or standard solutions are added in duplicate into the microwells along with a fixed volume of a specific rabbit anti-BLG antibody solution. With increased concentrations of BLG and antigenic BLG-fragments in the sample, the amount of anti-

BLG antibodies binding to BLG attached to the well will decrease. After washing, the amount of bound antibodies is determined by reaction with peroxidase conjugated anti-rabbit globulin.

Unbound conjugate is removed by washing and peroxidase activity is determined by addition of an enzyme substrate (urea peroxide) which develops a blue colour in presence of a chromogen (tetramethylbenzidine). Addition of the stop solution causes a color change from blue to yellow. The colour development is inversely proportional to the original BLG concentration in the standard solution and inversely proportional to the original BLG antigenicity (reactivity) of the sample solution. Semi-quantitation is performed by comparing absorbances at 450 nm of sample solutions with the calibration curve.

The BLG reactivity of protein hydrolysates (= BLG and antigenic BLG-fragments) is expressed as mg BLG equivalents/g protein.

Milk or whey contaminations traced by this method are expressed as mg BLG/kg product.

4 CHEMICAL AND MATERIALS

Commercial references are only a guideline. Numbers in the margin refer either to the Merck's chemicals and reagents catalogue or to that of Nestlé laboratory material

4.1 Chemicals

Before using chemicals refer to the Sigma/Aldrich Guide to Chemical Safety and/or other adequate manuals or safety data sheets approved by your local authorities.

- RIDASCREEN β-Lactoglobulin Cat. N°.R4901; r-Biopharm GmbH, Darmstadt, Germany

4.2 Materials

252703 - Disposable test tubes, polystyrene, 12 ml, with polyethylene stopper, Millian SA, Geneva
304103 - Volumetric flask 25 ml; RN 10/19
334802 - Micropipettes, adjustable, e.g. Socorex, 50-200 µl
334803 - idem, 200-1 000 µl
334804 - PP tips, yellow, 5-200 µl
334805 - PP tips blue, 200-1 000 µl
334901 - idem, 0,5-5 ml
334902 - PP tips white, 0,5-5 ml
804921 - Magnetic stirring bar, 5 x 20 mm
904201 - Centrifuge, Heraeus Labofuge
904203 - Rotor and support for tubes 4x15/7 ml
918401 - Microplate Reader, MR 5 000, Dynatech
918501 - Multi-Reagent Microplate Washer, Dynatech
918601 - Orbital plate shaker, Luckham 802, Brouwer, Switzerland
- Multipipette 4780, Eppendorf
- Combitips for Multipipette 4780, 5 ml, Eppendorf

- Combitips for Multipipette 4780, 2,5 ml, Eppendorf
- Combitips for Multipipette 4780, 1,0 ml, Eppendorf
- Microtest III tissue culture lids, Falcon 3071, Becton Dickinson

5 PREPARATION AND CHECK OF REAGENTS

5.1 BLG Assay Kit

Follow storage instructions of the supplier. Do not use a kit beyond its expiry date.
Remove all reagents from the boxes and allow them to reach room temperature before starting the assay.

5.1.1 Sample- and antibody dilution buffer and washing buffer

Prepare the volume required for the number of test wells needed by diluting the buffer concentrate 1 + 9 with water.

5.1.2 BLG Standards

The BLG standard solutions (0, 10, 30, 90, 270, 810 ng/ml) are supplied ready to use.

5.1.3 Anti-BLG antibody

The anti-BLG antibody is provided as a concentrate. Just before use, shake carefully the bottle, then prepare the needed amount by diluting the concentrate 1 + 10 in buffer (e. g. 200 µl antibody + 2 ml buffer is sufficient for 4 microtiter strips).

5.1.4 Anti-rabbit peroxidase conjugate

The anti-rabbit peroxidase conjugate is supplied pre-diluted in buffer. No preparation is necessary other than gently mixing the contents by repeated inversion.

5.1.5 Substrate

The substrate, which contains urea peroxidase, is supplied ready to use. No preparation is necessary other than gently mixing the contents by repeated inversion.

5.1.6 Chromogen (TMB)

The chromogen solution is supplied ready to use. No preparation is necessary other than gently mixing the contents by repeated inversion.

5.1.7 Stop reagent (1 M sulfuric acid)

The stop solution is supplied ready for use. No preparation is necessary other than gently mixing

6 SAMPLE PREPARATION

6.1 Preparation of the test portion and dilution of milk, whey and casein hydrolysate based infant formulas

6.1.1 Moderately hydrolysed

Powdered products

Into a 50 ml beaker, weigh a test portion corresponding to 125 mg protein equivalents. Determine proteins previously according to LI-00.556. E.g. for a powder with 11,0 % protein equivalents, weigh 1136 mg.

By means of a pipette, add 25 ml of 50 °C warm water and stir for 30 minutes.

Working dilution (40 x): Into a disposable test tube pipette 100 µl of this solution to 3 900 µl of buffer. This solution contains 0,1 mg protein/ml.

Liquid products

Into a 25 ml volumetric flask, weigh a test portion corresponding to 125 mg protein equivalents.

Make up to the mark with water. Dilute as outlined under 6.1.1.

6.1.2 Extensively hydrolysed

Weigh and dissolve test portion as outlined under 6.1.1, but dissolve in working diluent solution (5.1.1) instead of water. Centrifuge during 10 min at 700 *g*.

Working dilution (5 x, 10 x): Into two disposable test tubes introduce, by means of a pipette, each 100 µl of this solution and add 400 µl and 900 µl of buffer respectively. Perform immunoassay on both solutions containing 1,0 and 0,5 mg protein/ml respectively.

6.2 Preparation of the test portion and dilution of infant cereals and similar products

6.2.1 Powders (including Siniac)

Into a 50 ml Erlenmeyer flask, weigh to the nearest 10 mg about 2 g of sample. Add 25 ml of diluent buffer (5.1.1). Homogenise for 2 min with a Polytron-type homogeniser. Centrifuge the extract during 10 min. at 500 *g*. Transfer the supernatant into a test tube. Dilute 5 and 10 times (1+4 and 1+9) with the diluent solution (5.1.1).

6.2.2 Liquid samples

Into a 50 ml Erlenmeyer flask pipette a volume (x ml) corresponding to about 2 g of dry matter and weigh to the nearest 10 mg. Add (25 – x) ml of diluent buffer and continue as outlined for powders (6.2.1)

Note:
If in a sample solution more than 90 ng BLG/ml are measured, dilute with buffer in order to reach the working range of the standard curve (10 – 90 ng BLG/ml).

7 IMMUNOASSAY

Prepare the number of 8-well strips required for your analysis: all standard, reference sample, and sample dilutions must be run in duplicates.

7.1 Addition of antigen solutions

Dispense in duplicate 50 µl of each standard, sample dilutions and maximum binding to separate wells.

7.2 Addition of specific antibody solution

Add immediately 50 µl of rabbit anti-BLG to each well and mix thoroughly.
Caution: Do not allow the tip of the pipette to come in contact with the solution already in the microwells!

7.3 Incubation

Incubate the covered plate for 2 hours at room temperature, without shaking.

7.4 Washing

Using a microwell washer, aspirate the contents of the wells and fill with buffer wash solution (5.1.1). Repeat the operation 2 more times. Finally aspirate the contents of the wells. Tap the plate upside down on several layers of absorbent tissue to remove residual droplets of the wash solution and bubbles.

Note:
When inverting the plate be sure to squeeze the plastic frame at the centre of the long edges to prevent the strips from falling out of the frame.

7.5 Addition of conjugate

Using a repeating dispenser add 100 µl anti-rabbit peroxidase conjugate to each well. Complete promptly without interruption and mix thoroughly.

7.6 Incubation

Incubate the covered plate 30 minutes at room temperature without shaking.

7.7 Washing

Using a microwell washer, aspirate the contents of the wells and fill with buffer wash solution (5.1.1). Repeat the operation 2 more times. Finally aspirate the contents of the wells. Tap the plate upside down on several layers of absorbent tissue to remove residual droplets of the wash solution and bubbles.

Note:
When inverting the plate be sure to squeeze the plastic frame at the centre of the long edges to prevent the strips from falling out of the frame.

7.8 Addition of substrate and chromogen

Using a repeating dispenser, add 50 µl of substrate and 50 µl of chromogen to each well. Complete promptly without interruption and mix thoroughly.

7.9 Colour development

Incubate the covered plate 15 minutes at room temperature in the dark.

7.10 Addition of stop solution

Using a repeating dispenser add 100 µl stop solution to each well. Complete promptly without interruption. Mix plate gently for ten seconds.

7.11 Spectrophotometric measurement

Use a microplate reader fitted with a 450 nm filter. Measure the absorbance of each well against air.

7.12 Time of analysis

Preparation of samples:	Approx. 1h to 3h (depending on the number of samples)
Immunoassay:	Approx 3h 30 min.

8 CALCULATION, EXPRESSION AND INTERPRETATION OF THE RESULTS

8.1 Calibration curve

Generally, it is not necessary to draw calibration curves manually. Microplate readers, such as the Microplate Reader MR 5 000, from Dynatech, are already equipped with appropriate software that allows printing the calibration curve and direct calculation of results in ng BLG/ml.

A smooth curve should be obtained on a semilogarithmic plot (spline with tails, see enclosure 3), otherwise the assay must be repeated. If the O.D. of one single well is an outlier, that generates an abnormal curve, it can be eliminated, and the better fitting value of the duplicate be maintained. The O.D. of the maximum binding should be greater than 1,2 and smaller than 2,5.

Read the BLG concentration of the test portion from the calibration curve and determine the mean value. BLG concentrations < 10 ng/ml should be considered as not detectable under the conditions of the test. BLG concentrations > 90 ng/ml should be considered as being outside the measuring range. If in such a case quantification of BLG is required, dilute the sample solution accordingly and repeat the whole procedure.

8.2 Calculation

Protein hydrolysate based products

The BLG antigenicity, expressed in mg BLG equivalents/g protein, is equal to:

$$\frac{c \cdot d}{w \cdot 1000}$$

where:

c = mean BLG concentration of the sample, read from the calibration curve, in ng/ml.
d = dilution of the test portion (e.g.: 25 x 40 = 1 000, according to 6.1.1).
w = weight of the test portion, expressed in mg protein (generally: 125).

Infant cereals and similar products:

Express the results in mg BLG/kg product according to the following formula:

$$\frac{c \cdot d}{w}$$

where:

c = mean BLG concentration of the sample, read from the calibration curve, in ng/ml
d = dilution of the test portion (e.g.: 25 x 5 = 125, when diluting the sample extract 5 times).
w = weight of the test portion, expressed in mg (2 000).

8.3 Expression of results

Express the results with 2 significant digits.

9 PERFORMANCE CHARACTERISTICS

9.1 Detection limit

When working according to 6.1.1 (d = 1 000 x), the detection limit is 0,08 mg BLG equivalents/g protein.

When working according to 6.1.2 (d = 125 x and 250 x), the detection limit is 0,01 and 0,02 mg BLG equivalents/g protein, respectively.

When working according to 6.2 (d = 125 x and 250 x), the detection limit is 0,63 and 1,3 mg BLG/kg product, respectively.

9.2 Repeatability

The difference between the O.D. of duplicates analysed (same sample extraction), must not exceed 7,5 % of their average, which corresponds to the repeatability limit at 95 % confidence level (robust statistics).

10 ANALYTICAL FLOW SHEET

See Enclosure 2.

11 INTERNAL CONTROL PLAN

11.1 Reference sample

Include in each series of analysis the quantitation of a reference sample with a known BLG antigenicity. Stored at - 20 °C, this sample can be kept for at least 18 months.

12 REFERENCES

12.1 Bibliography

RE-RD010026: Determination of β-lactoglobulin in milk powders and similar products by ELISA by C. Martín-Hernández and H. Weymuth (2001-02-20).

12.2 LIs mentioned

LI-08.084: Determination of β-lactoglobulin by ELISA

LI-00.556: Total nitrogen according to Kjeldahl with Büchi.

13 APPROVAL OF THE METHOD

Approved by	Initials	Date
Author	NHc	21.02.01
Group Leader	CK	21.02.01
Head of Department	CD	22/02/01
CT-QM	RAS	22.2.2001

14 ENCLOSURES

1 Analytical flow sheet
2 Analytical flow sheet - Blank form
3 Standard curve

Enclosure 1

ANALYTICAL FLOW SHEET

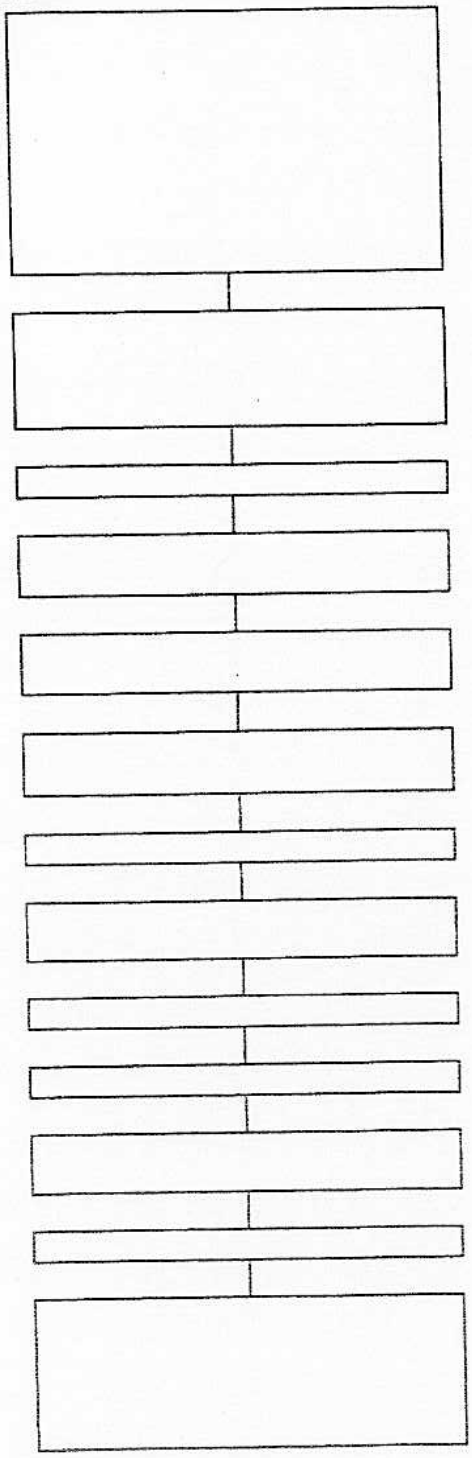
Steps	Critical points
SAMPLE PREPARATION *Hydrolysated infant formulas* : weigh 125 mg protein. Dissolve with 50 °C warm water or buffer (5.1.1). *Infant cereals, etc.*: weigh 2 g sample and homogenise with 25 ml buffer. Dilute sample solutions with buffer (5.1.1).	Solvent depending on sample type. Dilute sample solutions with dilution buffer (5.1.1) as follows: 40 x for moderately hydrolysed formulas. 5 x and 10 x for extensively hydrolysed infant formulas and infant cereals.
IMMUNOASSAY Dispense in duplicate 50 µL per well : Test portion and standard solutions Add 50 µl anti-BLG (5.1.3) to each well	Do not touch solution in microwells with pipette tip.
Incubate for 2 h	Room temperature, without shaking.
Rinse 3 x with buffer (5.1.1)	
Add 100 µl PEROXIDASE CONJUGATE (5.1.4) to each well	
Incubate for 30 minutes	Room temperature, without shaking.
Rinse 3 x with buffer (5.1.1)	
Add 50 µl SUBSTRATE(5.1.5) to each well	
Add 50 µl TMB (5.1.6) to each well	
Incubate 15 minutes	Room temperature. Adjust incubation time, if required.
Add 100 µl STOP REAGENT (5.1.7) to each well	
Read O.D. at 450 nm	
EVALUATION Calculate and express results in mg BLG equivalents/g protein for protein hydrolysates and in mg BLG/kg product for infant cereals	

Enclosure 2

ANALYTICAL FLOW SHEET - BLANK FORM

Steps

Critical points



Enclosure 3

Representative standard curve of BLG with the average characteristics of 8 different experiments.